NEW EUROPEAN PATENT SPECIFICATION

(12)

(54) TUMOUR NECROSIS FACTOR BINDING LIGANDS

BINDELIGANDE FÜR TUMORNEKROSISFAKTOR

LIGANDS DE LIAISON DU FACTEUR DE NECROSE DE TUMEURS

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• The Journal of Immunology, Vol.141, No.2, issued July 15,1988 (U.S.A.) SHALABY, M.R., et al, "The Involvement of human tumor necrosis factors -alfa and -beta in the mixed lymphocyte reaction" see page 499.
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• PATENTS ABSTRACTS OF JAPAN, C-678, page 112, JP,A, 1-268645 (TEIJIN LTD) 26 October 1989 (26.10.89).

Korrelation
Epitope - biological
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in the r gions of residues 22-31 and 146-157 would be expect d to have the same effect on the bioactivity of TNF as MAb 37 and a ligand which binds to TNF predominately in the regions of residues 22-40, 69-97, 105-128 and 135-155 would be expected to have the same effect on the bioactivity of TNF as MAb 53

[0080] The present inventors hav quite cl arly shown that the bioactivity of TNF can b alt red by the binding f a ligand to the TNF, and that the effect on the bioactivity is a function of the specificity of the ligand. For example, the binding of MAb 32 to TNF in the regions of residues 1-26, 117-128 and 141-153 results in the induction of endothelial procoagulant activity of the TNF and binding of TNF to receptors on endothelial cells being inhibited; the induction of tumour fibrin deposition and tumour regression activities of the TNF being enhanced; the cytotoxicity being unaffected and the tumour receptor binding activities of the TNF being unaffected or enhanced. It is believed that this effect on the bioactivity of the TNF may be due to the prevention of the binding of the epitope of the TNF recognised by MAb 32 to naturally occurring biologically active ligands. Accordingly, it is believed that a similar effect to that produced by MAb 32 could also be produced by a ligand which binds to a region of TNF in a manner such that the epitope recognised by MAb 32 is prevented from binding to naturally occurring biologically active ligands. This prevention of binding may be due to steric hindrance or other mechanisms.

[0081] Accordingly, it is intended that the prevention of the binding of epitopes recognised by the various monoclonal antibodies described herein to naturally occurring biologically active ligands is within the scope of the present invention.

Claims

Claims for the following Contracting States : CH, DE, DK, FR, GB, IT, LI, NL, SE

1. An antibody or antibody fragment capable of binding to TNF, the antibody or antibody fragment being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited, the antibody or antibody fragment binding to the TNF such that the epitope of the TNF defined by the topographic region of 1-18, 58-65, 115-125 and 138-149, or the topographic region of residues 1-18 and 108-128, or the topographic region of residues 56-79, 110-127 and 135-155, or the topographic region of residues 1-30, 117-128 and 141-153, or the topographic region of residues 1-18, or the topographic region of residues 22-40, 49-97, 110-127 and 136-153, or the topographic region of residues 1-20 and 76-90, or the topographic region of residues 22-40, 69-97, 105-128 and 135-155 is substantially prevented from binding to naturally occurring biologically active ligands.

2. An antibody or antibody fragment as claimed in claim 1 in which the antibody or antibody fragment is further characterised in that when it binds to TNF the tumour regression, induction of tumour fibrin deposition, cytotoxicity and receptor binding activites of the TNF are inhibited, the antibody or antibody fragment binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 1-18, 58-65, 115-125 and 138-149, or the topographic region of residues 1-18 and 108-128, or the topographic region of residues 56-79, 110-127 and 135-155 is substantially prevented from binding to naturally occurring biologically active ligands.

3. An antibody or antibody fragment as claimed in claim 1 or 2 in which the antibody or antibody fragment is a monoclonal antibody selected from MAb 1 (ECACC 89080301), MAb 54 (ECACC 89083103) and MAb 47 (ECACC 89121402) or a fragment thereof.

4. The use of an antibody or antibody fragment as claimed in claim 2 or 3 in the preparation of an agent for the treatment of toxic shock.

5. An antibody or antibody fragment as claimed in claim 1 in which the antibody or antibody fragment is further characterised in that when it binds to TNF the binding of the TNF to receptors on endothelial cells is inhibited, the induction of tumour fibrin deposition and tumour regression activities of the TNF are enhanced; the cytotoxicity of the TNF is unaffected; and the tumour receptor binding activity of the TNF is unaffected or enhanced; the antibody or antibody fragment binding to TNF such that the epitope of the TNF defined by the topographic region of residues 1-30, 117-128 and 141-153 or the topographic region of residues 1-18 is substantially prevented from binding to naturally occurring biologically active ligands.

6. An antibody or antibody fragment as claimed in claim 5 in which the antibody or antibody fragment binds to human TNF in the topographic regions of residues 1-26, 117-128 and 141-153.

7. An antibody or antibody fragment as claimed in claim 6 in which the antibody or antibody fragment is MAb 32

(ECACC 89080302) or a fragment thereof.

8. An antibody or antibody fragment as claimed in claim 5 in which the antibody or antibody fragment binds to r sidues 1-18 of human TNF (peptide 301).

9. An antibody or antibody fragment as claimed in claim 1 in which the antibody or antibody fragment is further characterised in that when it binds to TNF the cytotoxicity and tumour regression activities of the TNF are unaffected; the induction of tumour fibrin deposition activity of the TNF is inhibited and the receptor binding activities of the TNF are unaffected; the antibody or antibody fragment binding to TNF such that the epitope of the TNF defined by the topographic regions of residues 22-40, 49-97, 110-127 and 136-153 is substantially prevented from binding to naturally occurring biologically active ligands.

10. An antibody or antibody fragment as claimed in claim 9 in which the antibody or antibody fragment is MAb 42 (ECACC 89080304) or a fragment thereof.

11. The use of an antibody or antibody fragment as claimed in any one of claims 5 to 10, in the preparation of an agent for the treatment of tumours inhibited by the action of TNF.

12. The use as claimed in claim 11 in which the tumour is selected from the group consisting of melanoma, breast and bladder carcinomas.

13. A product containing an antibody or antibody fragment as claimed in any one of claims 5 to 10 and a cytotoxic drug for simultaneous, sequential or separate administration in cancer therapy.

14. A product as claimed in claim 13 in which the cytotoxic drug is selected from the group consisting of vinblastic, acyclovir, interferon alpha, IL-2, actinomycin D, AZT, adriamycin, mytomycin C, cytosine arabinoside, dounorubicin, cis-platin, vincristine, 5-flurouracil and bleomycin.

15. An antibody or antibody fragment as claimed in claim 1 in which the antibody or antibody fragment is further characterised in that when it binds to TNF the tumour fibrin deposition activity of the TNF is enhanced and the cytotoxicity, tumour regression, and receptor binding activities of the TNF are inhibited, the antibody or antibody fragment binding to TNF such that the epitope of the TNF defined by the topographic regions of residues 1-20 and 76-90 is substantially prevented from binding to naturally occurring biologically active ligands.

16. An antibody or antibody fragment as claimed in claim 15 in which the antibody or antibody fragment binds to TNF in the regions of residues 1-18 and 76-90.

17. An antibody or antibody fragment as claimed in claim 15 or 16 in which the antibody or antibody fragment is MAb 21 (ECACC 90012432) or a fragment thereof.

18. An antibody or antibody fragment as claimed in claim 1 in which the antibody or antibody fragment is further characterised in that when it binds to TNF the tumour fibrin deposition activity of the TNF is unaffected and the cytotoxicity, tumour regression and receptor binding activities of the TNF are inhibited, the antibody or antibody fragment binding to TNF such that the epitope of the TNF defined by the topographic regions of residues 22-40, 69-97, 105-128 and 135-155 is substantially prevented from binding to naturally occurring biologically active ligands.

19. An antibody or antibody fragment as claimed in claim 18 in which the antibody or antibody fragment is MAb 53 (ECACC 90012433) or a fragment thereof.

20. An antibody or antibody fragment capable of binding to human TNF, such that when it binds to TNF the tumour fibrin deposition activity of the TNF is enhanced; the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression and receptor binding activities of the TNF are inhibited, characterised in that when the antibody or antibody fragment binds to the TNF, the epitope of the TNF defined by the topographic regions of residues 12-22, 36-45, 96-105 and 132-157 is substantially prevented from binding to naturally occurring biologically active ligands, and/or the antibody or antibody fragment binds to human TNF in the topographic regions of residues 12-22, 36-45, 96-105 and 132-157.

21. An antibody or antibody fragment as claimed in claim 20 in which the antibody or antibody fragment is MAb 25 (ECACC 89121401) or a fragment thereof.

22. An antibody or antibody fragment capable of binding to human TNF, such that the tumour fibrin deposition, the induction of endothelial procoagulant, cytotoxicity, tumour regression and receptor binding activities of the TNF are unaffected, characterised in that when the antibody or antibody fragment binds to the TNF, the epitope of the TNF defined by the topographic regions of residues 22-31 and 146-157 is substantially prevented from binding to naturally occurring biologically active ligands, and/or the antibody or antibody fragment binds to human TNF in the topographic regions of residues 22-31 and 146-157.

23. An antibody or antibody fragment as claimed in claim 22 in which the antibody or antibody fragment is MAb 37 (ECACC 89090303) or a fragment thereof.

24. An antibody or antibody fragment capable of binding to human TNF, such that the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression, tumour fibrin deposition and receptor binding activities of the TNF are inhibited characterised in that when the antibody or antibody fragment binds to the TNF, the epitope of the TNF defined by the topographic regions of residues 22-40 and either 49-98 or 70-87 is substantially prevented from binding to naturally occurring biologically active ligands and/or the antibody or antibody fragment binds to human TNF in the topographic region of residues 22-40 and either 49-98 or 70-87, wherein the antibody is not antibody AM-195 secreted by cell line ECACC 87050801.

25. A composition comprising TNF and an antibody or antibody fragment as claimed in any one of claims 1-3, 5-10 or 15-24, in which the ligand is bound to the TNF.

26. An antibody or antibody fragment as claimed in any of claims 1-3, 5-10 or 15-24 for use in medicine.

Claims for the following Contracting State : ES

1. A process comprising providing an antibody or antibody fragment capable of binding to TNF, the antibody or antibody fragment being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited, the antibody or antibody fragment binding to the TNF such that the epitope of the TNF defined by the topographic region of 1-18, 58-65, 115-125 and 138-149, or the topographic region of residues 1-18 and 108-128, or the topographic region of residues 56-79, 110-127 and 135-155, or the topographic region of residues 1-30, 117-128 and 141-153, or the topographic region of residues 1-18, or the topographic region of residues 22-40, 49-97, 110-127 and 136-153, or the topographic region of residues 1-20 and 76-90, or the topographic region of residues 22-40, 69-97, 105-128 and 135-155 is substantially prevented from binding to naturally occurring biologically active ligands.

2. A process comprising producing an antibody or antibody fragment as defined in claim 1 in which the antibody or antibody fragment is further characterised in that when it binds to TNF the tumour regression, induction of tumour fibrin deposition, cytotoxicity and receptor binding activites of the TNF are inhibited, the antibody or antibody fragment binding to the TNF such that the epitope of the TNF defined by the topographic regions of residues 1-18, 58-65, 115-125 and 138-149, or the topographic region of residues 1-18 and 108-128, or the topographic region of residues 56-79, 110-127 and 135-155 is substantially prevented from binding to naturally occurring biologically active ligands.

3. A process comprising producing an antibody or antibody fragment as defined in claim 1 or 2 in which the antibody or antibody fragment is a monoclonal antibody selected from MAb 1 (ECACC 89080301), MAb 54 (ECACC 89083103) and MAb 47 (ECACC 89121402) or a fragment thereof.

4. The use of an antibody or antibody fragment as defined in claim 2 or 3 in the preparation of an agent for the treatment of toxic shock.

5. A process comprising producing an antibody or antibody fragment as defined in claim 1 in which the antibody or antibody fragment is further characterised in that when it binds to TNF the binding of the TNF to receptors on endothelial cells is inhibited, the induction of tumour fibrin deposition and tumour regression activities of the TNF are enhanced; the cytotoxicity of the TNF is unaffected; and the tumour receptor binding activity of the TNF is

Des ripti n

[0001] The present invention relates to ligands which bind to human tumour necrosis factor alpha (TNF) in a manner such that upon binding the biological activity of TNF is modified. The type of modification shown here is distinct from previous descriptions of antibodies which bind to TNF alpha and inhibit all TNF alpha activity The new discovery shows how the different activities of TNF alpha can be selectively inhibited or enhanced. In addition, the pr sent invention relates to a composition comprising a molecule bound to TNF and to methods of therapy utilising TNF and molecules active against TNF.

[0002] Tumor necrosis factor alpha (TNF) is a product of activated macrophages first observed in the serum of ex-perimental animals presensitized with Bacillus Calmette-Guerin or Corynebacterium parvum and challenged with en-dotoxin (LPS). Following the systematic administration of TNF haemorrhagic necrosis was observed in some trans-plantable tumours of mice while in vitro TNF caused cytolytic or cytostatic effects on tumour cell lines

[0003] In addition to its host-protective effect, TNF has been implicated as the causative agent of pathological chang-es in septicemia, cachexia and cerebral malaria. Passive immunization of mice with a polyclonal rabbit serum against TNF has been shown to protect mice against the lethal effects of LPS endotoxin, the initiating agent of toxic shock, when administered prior to infection.

[0004] The gene encoding TNF has been cloned allowing the usefulness of this monokine as a potential cancer therapy agent to be assessed. While TNF infusion into cancer patients in stage 1 clinical trials has resulted in tumour regression, side-effects such as thrombocytopaenia, lymphocytopaenia, hepatotoxicity, renal impairment and hyper-tension have also been reported These quite significant side-effects associated with the clinical use of TNF are pre-dictable in view of the many known effects of TNF. some of which are listed in Table 1.

TABLE 1
BIOLOGICAL ACTIVITIES OF TNF
- ANTI-TUMOUR
- ANTI-VIRAL
- ANTI-PARASITE

FUNCTION

[0005]

cytotoxic action on tumour cells
pyrogenic activity
angiogenic activity
inhibition of lipoprotein lipase
activation of neutrophils
osteoclast activation
induction of endothelial, monocyte and tumour cell procoagulant activity
induction of surface antigens on endothelial cells
Induction of IL-6
induction of c-myc and c-fos
induction of EGF receptor
Induction of IL-1
induction of TNF synthesis
induction of GM-CSF synthesis
increased prostaglandin and collagenase synthesis
induction of acute phase protein C3

[0006] Of particular importance is the activation of coagulation which occurs as a consequence of TNF activation of endothelium and also peripheral blood monocytes. Disseminated intravascular coagulation is associated with toxic shock and many cancers Including gastro-intestinal cancer, cancer of the pancreas, prostate, lung, breast and ovary, melanoma, acute leukaemia, myeloma, myeloproliferative syndrome and myeloblastic leukaemia Clearly modifications of TNF activity such that tumour regression activity remains intact but other undesirable effects such as activation of coagulation are removed or masked would lead to a more advantageous cancer therapy. while complete abrogation of TNF activity is sought for successful treatment of toxic shock.

[0007] Segregation of hormonal activity through the use of site-specific antibodies (both polyclonal and monoclonal)

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can result in nhanced hormonal activity (Aston t al, 1989, Mol. Immunol. 26, 435). To date few attempts have been made to assign antigenicity or function to particular regions of the TNF molecule for which the three-dimensional structure is now known. Assignment of function to such regions would permit the d velopment of MAbs and other ligands of therap utic use. Polyclonal antibodies to amino acids 1 to 15 have been reported to block Hela R19 cell r ceptor

5   binding by TNF (Socher et al, 1987, PNAS 84, 8829) whilst monoclonal antibodies recognising undefined conformational epitopes on TNF have been shown to inhibit TNF cytotoxicity in vitro (Bringman and Aggarwal, 1987, Hybridoma 6. 489). However, the effects of these antibodies on other TNF activities is unknown.

[0008]   EP-A-0288088 discloses a limited number of monoclonal antibodies which are said to bind to particular epitopes of human TNF These epitopes are contained in the 68th to 97th, 7th to 37th. and 113th to 127th amino acids

10   of human TNF.

[0009]   EP-A-0260610 discloses monoclonal antibodies binding to human TNF including AM-195 secreted by cell line ECACC 87050801, which neutralises cytotoxic activity of human TNF.

[0010]   The present inventors have produced panels of monoclonal antibodies active against human TNF and have characterised them with respect to their effects on the anti-tumour effect of TNF (both in vitro and in vivo), TNF receptor

15   binding, activation of coagulation (both in vitro and in vivo) and defined their topographic specificities. This approach has led the inventors to show that different topographic regions of TNF alpha are associated with different activities. Therefore the inventors enable the identification of antibodies or ligands which selectively enhance or inhibit TNF alpha activity, thereby providing for improved therapeutic agents and regimes including TNF alpha.

[0011]   According to a first embodiment of the present invention, there is provided an antibody or antibody fragment

20   capable of binding to TNF, the antibody or antibody fragment being characterised in that when it binds to TNF the induction of endothelial procoagulant activity of the TNF is inhibited, the antibody or antibody fragment binding to the TNF such that the epitope of the TNF defined by the topographic region of 1-18, 58-65, 115-125 and 138-149, or the topographic region of residues 1-18 and 108-128, or the topographic region of residues 56-79, 110-127 and 135-155 or the topographic region of residues 1-30, 117-128 and 141-153, or the topographic region of residues 1-18, or the

25   topographic region of residues 22-40, 49-97, 110-127 and 136-153, or the topographic region of residues 1-20 and 76-90. or the topographic region of residues 22-40, 69-97, 105-128 and 135-155 is substantially prevented from binding to naturally occurring biologically active ligands.

[0012]   According to a second embodiment of the present invention there is provided an antibody or antibody fragment capable of binding to human TNF. such that when it binds to TNF the tumour fibrin deposition activity of the TNF is

30   enhanced; the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity. tumour regression and receptor binding activities of the TNF are inhibited, characterised in that when the antibody or antibody fragment binds to the TNF, the epitope of the TNF defined by the topographic regions of residues 12-22, 36-45, 96-105 and 132-157 is substantially prevented from binding to naturally occurring biologically active ligands, and/or the antibody or antibody fragment binds to human TNF in the topographic regions of residues 12-22, 36-45, 96-105 and

35   132-157.

[0013]   According to a third embodiment of the present invention, there is provided an antibody or antibody fragment capable of binding to human TNF, such that the tumour fibrin deposition, the induction of endothelial procoagulant, cytotoxicity, tumour regression and receptor binding activities of the TNF are unaffected, characterised in that when the antibody or antibody fragment binds to the TNF, the epitope of the TNF defined by the topographic regions of

40   residues 22-31 and 146-157 is substantially prevented from binding to naturally occurring biologically active ligands, and/or the antibody or antibody fragment binds to human TNF in the topographic regions of residues 22-31 and 146-157.

[0014]   According to a fourth embodiment of the present invention, there is provided an antibody capable of binding to human TNF, such that the induction of endothelial procoagulant activity of the TNF is unaffected and the cytotoxicity, tumour regression, tumour fibrin deposition and receptor binding activities of the TNF are inhibited characterised in

45   that when the antibody or antibody fragment binds to the TNF, the epitope of the TNF defined by the topographic regions of residues 22-40 and either 49-98 or 70-87 is substantially prevented from binding to naturally occurring biologically active ligands and/or the antibody or antibody fragment binds to human TNF in the topographic region of residues 22-40 and either 49-98 or 70-87, wherein the antibody is not antibody AM-195 secreted by cell line ECACC 87050801.

[0015]   Preferred aspects of the present invention are given in the dependent claims.

50   [0016]   The antibody or antibody fragment can be selected from the group consisting F(ab) fragments, restructured anti-bodies (CDR grafted humanised antibodies) single domain antibodies (dAbs) and single chain antibodies. However it is presently preferred that the antibody or antibody fragment is a monoclonal antibody or F(ab) fragment thereof.

[0017]   The present invention includes particular monoclonal antibodies or fragments thereof. These antibodies are

55   listed below :

A) A monoclonal antibody selected from the group consisting of the monoclonal antibodies designated MAb 1, MAb 47 and MAb 54 (see claim 3). Samples of the hybridoma cell lines which produce MAb 1, MAb 54 and MAb

47 have been deposit d with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, United Kingdom, MAb 1 was deposited on 3 August 1989 and accorded accession No. 89080301; MAb 54 was deposited on 31 August 1989 and accorded accession No. 89083103; MAb 47 was deposited on 14 December 1989 and accorded accession No. 89121402.

B) The monoclonal antibody designated MAb 42 (see claim 10). A sample of the hybridoma cell line producing MAb 42 was deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, United Kingdom on 3 August 1989 and was accorded accession No. 89080304.

C) The monoclonal antibody designed MAb 25 (see claim 21). A sample of the hybridoma cell line producing MAb 25 was deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, United Kingdom on 14 December 1989 and was accorded accession No.89121401.

D) The monoclonal antibody designated MAb 21 (see claim 17). A sample of the hybridoma cell line producing MAb 21 was deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, United Kingdom on 25 January 1990 and was accorded accession No. 90012432.

E) The monoclonal antibody designated MAb 53 (see claim 19). A sample of the hybridoma cell line producing MAb 53 was deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, United Kingdom on 25 January 1990 and was accorded accession No. 90012433.

F) The monoclonal antibody designated MAb 37 (see claim 23). A sample of the hybridoma cell line producing MAb 37 was deposited with the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, United Kingdom on 3 August 1989 and was accorded accession No. 89080303.

[0018] The biological activities of TNF referred to herein by the terms "Tumour Regression", "Induction of Endothelial Procoagulant", "Induction of Tumour Fibrin Deposition", "Cytotoxicity" and "Receptor Binding" are to be determined by the methods described below.

[0019] The term "single domain antibodies" as used herein is used to denote those antibody fragments such as described in Ward et al (Nature, 341, 544-546 (1989)) as suggested by these authors.

[0020] In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following example and accompanying figures in which:-

Fig. 1 shows the results of a titration assay with MAb 1 against TNF;
Fig. 2 shows TNF MAb 1 scatchard plot and affinity determination;
Fig. 3 shows the effect of anti-TNF monoclonal antibodies 1 and 32 on TNF cytotoxicity in WEHI-164 cells;
Fig. 4 shows the effect of MAb 1 on TNF-induced regression of a Meth A solid tumour;
Fig. 5 shows the effect of MAbs 1 and 25 on TNF-induced Meth A Ascites tumour regression;
Fig. 6 shows the effect of anti-TNF MAbs on induction of endothelial cell procoagulant activity by TNF;
Fig. 7 shows incorporation of labelled fibrinogen into tumours of tumour-bearing mice and the effect of anti-TNF MAbs:
Fig. 8 is a schematic representation of epitopes on TNF;
Fig. 9 shows the effect of anti-TNF MAbs on TNF-induced regression of WEHI-164 tumours;
Fig. 10 shows the enhancement of TNF regression activity by MAb 32 in two experiments:
Fig. 11 shows the enhancement of TNF-induced tumour regression by MAb 32 - dose response at day 1 and day 2:
Fig. 12 shows binding of radio labelled TNF to receptors on bovine aortic endothelial cells;
Fig. 13 shows receptor binding studies of TNF complexed with MAb 32 (—◇—), control antibody (—□—) and MAb 47 (—◁—) on melanoma cell line MM418E:
Fig. 14 shows receptor binding studies of TNF complexed with MAb 32 (—◇—), control antibody (—□—) and MAb 47 (—◁—) on melanoma cell line IGR3:
Fig. 15 shows receptor binding studies of TNF complexed with MAb 32 (—◇—), control antibody (—□—) and MAb 47 (—◁—) on bladder carcinoma cell line 5637;
Fig. 16 shows receptor binding studies of TNF complexed with MAb 32 (—◇—), control antibody (—□—) and

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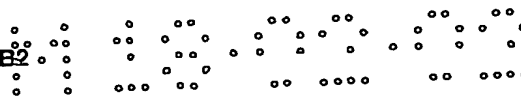
MAb 47 (—▱—) on breast carcinoma cell line MCF7;
Fig. 17 shows receptor binding studies of TNF complexed with MAb 32 (—◇—), control antibody (—▱—) and
MAb 47 (—△—) on colon carcinoma cell lin B10:
Fig. 18 shows the effect on TNF-mediated tumour regression in vivo by MAb 32 (▱) control MAb (▱) and
MAb 47 (*):
Fig. 19 shows the effect on TNF-mediated tumour regression in vivo by control MAb, MAb 32 and univalent FAb'
fragments of MAb 32;
Fig. 20 shows the effect on TNF induced tumour regression by control MAb (▱), MAb 32 (▰) and peptide
301 antiserum (▱):
Fig. 21 shows MAb.32 reactivity with overlapping peptides of 10 AA length; and
Fig. 22 shows a schematic three dimensional representation of the TNF molecule.
Fig. 23 shows topographically the region of residues 1 - 20, 56 - 77, 108 - 127 and 138 - 149:
Fig. 24 shows topographically the region of residues 1 - 18 and 108..-128:
Fig. 25 shows topographically the region of residues 56-79, 110 - 127 and 136 - 155;
Fig. 26 shows topographically the region of residues 1 - 26, 117 - 128 and 141 - 153;
Fig. 27 shows topographically the region of residues 22 - 40, 49 - 97, 110 - 127 and 136 - 153;
Fig. 28 shows topographically the region of residues 12 - 22, 36 - 45, 96 - 105 and 132 - 157;
Fig. 29 shows topographically the region of residues 1 - 20 and 76 - 90;
Fig. 30 shows topographically the region of residues 22 - 40, 69 - 97, 105 - 128 and 135 - 155;
Fig. 31 shows topographically the region of residues 22 - 31 and 146 - 157:
Fig. 32 shows topographically the region of residues 49 - 98; and
Fig. 33 shows topographically the region of residues 22-40 and 70-87.

Animals and Tumour Cell Lines

[0021]   In all experiments BALB/C female mice aged 10-12 weeks obtained from the CSIRO animal facility were used.
Meth A solid tumour and Meth A ascites tumour cell lines were obtained from the laboratory of Dr. Lloyd J. Old (Sloan
Kettering Cancer Centre) and the WEHI-164 fibrosarcoma line was obtained from Dr. Geeta Chauhdri (John Curtin
School of Medical Research, Australian National University).

Fusions and Production of Hybridomas

[0022]   Mice were immunised with 10 ug human recombinant TNF intra-peritoneally in Freund's complete adjuvant.
One month later 10 ug TNF in Freund's incomplete adjuvant was administered. Six weeks later and four days prior to
fusion selected mice were boosted with 10 ug TNF in PBS. Spleen cells from immune mice were fused with the myeloma
Sp2/0 according to the procedure of Rathjen and Underwood (1986, Mol. Immunol. 23, 441). Cell lines found to secrete
anti-TNF antibodies by radioimmunoassay were subcloned by limiting dilution on a feeder layer of mouse peritoneal
macrophages. Antibody subclasses were determined by ELISA (Misotest, Commonwealth Serum Laboratories).

Radioimmunoassay

[0023]   TNF was iodinated using lactoperoxidase according to standard procedures. Culture supernatants from hy-
bridomas (50 ul) were incubated with 1251 TNF (20,000 cpm in 50 ul) overnight at 4°C before the addition of 100 ul
Sac-Cel (donkey anti-mouse/rat immunoglobulins coated cellulose, Wellcome Diagnostics) and incubated for a further
20 minutes at room temperature (20°C). Following this incubation 1 ml of PBS was added and the tubes centrifuged
at 2,500 rpm for 5 minutes. The supernatant was decanted and the pellet counted for bound radioactivity.

Antibody-Antibody Competition Assays

[0024]   The comparative specificites of the monoclonal antibodies were determined in competition assays using either
immobilized antigen (LACT) or antibody (PACT) (Aston and Ivanyi, 1985, Pharmac. Therapeut. 27, 403).

PACT

[0025]   Flexible microtitre trays were coated with monoclonal antibody (sodium sulphate precipitated globulins from
mouse ascites fluid, 100 micrograms per ml in sodium bicarbonate buffer, 0.05M, pH 9.6) overnight at 4°C prior to
blocking non-specific binding sites with 1% bovine serum albumin in PBS (BSA/PBS). The binding of 1251 TNF to
immobilised antibody was determined in the presence of varying concentrations of a second anti-TN F monoclonal

antibody. Antibody and TNF were added simultaneously and incubated for 24 hours prior to washing with PBS (4 times) and counting wells for bound radioactivity. 100% binding was determined in the absence of heterologous monoclonal antibody while 100% competition was determin d in the presence of excess homologous monoclonal antibody. All dilutions were prepared in BSA/PBS.

LACT

[0026] The binding of protein A purified, radiolabelled monoclonal antibodies to TNF coated microtitre wells was determined in the presence of varying concentrations of a second monoclonal antibody. Microtitre plates were coated with TNF (50 micrograms per ml) as described above. Quantities of competing antibodies (50 microlitres) were pre-incubated on plates for 4 hour at 4°C prior to addition of 125I monoclonal antibody (30,000 cpm) for a further 24 hours. Binding of counts to wells was determined after four washes with PBS. 100% binding was determined in the absence of competing antibody while 100% competition was determined in the presence of excess unlabelled monoclonal antibody.

WEHI-164 Cytotoxicity Assay

[0027] Bioassay of recombinant TNF activity was performed according to Espevik and Nissen-Meyer (1986, J. Immunol. Methods 95, 99). The effect of the monoclonal antibody on TNF activity was determined by the addition of the monoclonal antibody to cell cultures at ABT90.

Tumour Regression Experiments

[0028] Modulation of TNF-induced tumour regression activity by monoclonal antibodies was assessed in three tumour models: the subcutaneous tumours WEHI-164 and Meth A sarcoma and the ascitic Meth A tumour. Subcutaneous tumours were induced by the injection of approximately 5×10^5 cells. This produced tumours of between 10 - 15 mm approximately 14 days later. Mice were injected intra-peritoneally with human recombinant TNF (10 micrograms) plus monoclonal antibody (200 microlitres ascites globulin) for four consecutive days. Control groups received injections of PBS alone or TNF plus monoclonal antibody against bovine growth hormone. At the commencement of each experiment tumour size was measured with calipers in the case of solid tumours or tumour-bearing animals weighed in the case of ascites mice. These measurements were taken daily throughout the course of the experiment.

Radio-Receptor Assays

[0029] WEHI-164 cells grown to confluency were scrape harvested and washed once with 1% BSA in Hank's balanced salt solution (HBSS, Gibco). 100 ul of unlabelled TNF (1-10,000 ng/tube) or monoclonal antibody (10 fold dilutions commencing 1 in 10 to 1 in 100,000 of ascitic globulin) was added to 50ul 125I TNF (50,000 cpm). WEHI cells were then added (200 microlitres containing 2×10^6 cells). This mixture was incubated in a shaking water bath at 37°C for 3 hours. At the completion of this incubation 1 ml of HBSS was added and the cells spun at 16,000 rpm for 30 seconds. The supernatant was discarded and bound 125I TNF in the cell pellet counted. All dilutions were prepared in HBSS containing 1% BSA.

Procoagulant Induction by TNF on Endothelial Cells

[0030] Bovine aortic endothelial cells (passage 10) were grown in RPMI-1640 containing 10% foetal calf serum (FCS), penicillin, streptomycin, and 2-mercaptoethanol at 37°C in 5% CO_2. For induction of procoagulant activity by TNF the cells were trypsinised and plated into 24-well Costar trays according to the protocol of Bevilacqua et al., 1986 (PNAS 83, 4533). TNF (0-500 units/culture) and monoclonal antibody (1 in 250 dilution of ascitic globulin) was added after washing of the confluent cell monolayer with HBSS. After 4 hours the cells were scrape harvested, frozen and sonicated. Total cellular procoagulant activity was determined by the recalcification time of normal donor platelet-poor plasma performed at 37°C, 100 microlitres of citrated platelet-poor plasma was added to 100 ul of cell lysate and 100 ul of calcium chloride (30mM) and the time taken for clot formation recorded. In some experiments tumour cell culture supernatant was added to endothelial cells treated with TNF and/or monoclonal antibody (final concentration of 1 in 2).

Incorporation of 125I Fibrinogen into Tumours of Mice Treated with TNF and Monoclonal Antibody

[0031] In order to examine the effect of TNF and monoclonal antibodies on fibrin formation in vivo, BALB/c mice were injected subcutaneously with WEHI-164 cells (10^5 cells/animal). After 7 - 14 days, when tumours reached a size

of approximately 1 cm in diamet r, animals were injected intra-peritoneally with TNF (10 ug/animal) and 125I human fibrinogen (7.5ug/animal, 122uCi/mg Amersham) either alone or in the presence of monoclonal antibody to human TNF (200ul/animal ascitic globulin). Monoclonal antibody against bovine growth hormone was used as control mono-clonal antibody. Two hours after TNF infusion incorporation of 125I fibrinogen into mouse tissue was determined by removing a piece of tissue, weighing it and counting the sample in a gamma count r.

[0032] In all 13 monoclonal antibodies reacting with human TNF were isolated. These monoclonal antibodies were designated MAb 1, MAb 11, MAb12, MAb 20, MAb 21, MAb 25, MAb 31, MAb 32, MAb 37, MAb 42, MAb 47, MAb 53 and MAb 54. The effect of these monoclonal antibodies on the bioactivity of human TNF is set out in Table 2.

[0033] As can be seen from Table 2, whilst some monoclonal antibodies inhibit both anti-tumour activity and activation of coagulation by human TNF (MAb 1, 47 and 54) not all antibodies which inhibit the anti-tumour activity inhibit activation of coagulation either in vitro or in vivo (MAb 11, 12, 25 and 53). Indeed MAb 21 which inhibited tumour regression enhanced the activation of coagulation in vivo.

TABLE 2

EFFECT OF MONOCLONAL ANTIBODIES ON TNF BIOACTIVITY													
MONOCLONAL ANTIBODY													
TNF BIOACTIVITY	1	11	12	20	21	25	31	32	37	42	47	53	54
Cytotoxicity	-	-	-	0	-	-	0	0	0	0	-	-	-
Tumour Regression	-	-	-	0	-	-	0	+	0	0	-	-	-
Induction of Procoagulant (Endothelial)	-	0	0	-	-	0	0	-	0	-	-	-	-
Fibrin Deposition (tumour)	-	-	-	+	+	+	+	+	0	-	-	0	-
Receptor Binding (WEHI-164)	-	-	-	0	-	-	0	+/0*	0	0	-	-	-

+ Enhancement 0 No effect - Inhibition * Depending on MAb concentration in the case of WEHI-164 tumour cells and tumour type (see Figs. 3,13-17).

[0034] MAbs 1, 47 and 54, which have been shown in competition binding studies to share an epitope on TNF, can be seen to have highly desirable characteristics in treatment of toxic shock and other conditions of bacterial, viral and parasitic infection where TNF levels are high requiring complete neutralisation of TNF. Other monoclonal antibodies such as MAb 32 are more appropriate as agents for coadministration with TNF during cancer therapy since they do not inhibit tumour regression but do inhibit activation of coagulation. This form of therapy is particularly indicated in conjunction with cytotoxic drugs used in cancer therapy which may potentiate activation of coagulation by TNF (e.g. vinblastin, acyclovir, IFN alpha, IL-2, actinomycin D, AZT, radiotherapy, adriamycin, mytomycin C, cytosine arabinoside, dounorubicin, cis-platin, vincristine, 5-flurouracil, bleomycin, (Watanabe N et al 1988 5 Immunopharmacol. Immunotoxicol. 10 117-127) or in diseases where at certain stages TNF levels are low (e.g. AIDS) and where individuals may have AIDS associated cancer e.g. Kaposi sarcoma, non-Hodgkins lymphoma and squamous cell carcinoma.

[0035] Monoclonal antibody MAb 1 has been found to have the following characteristics:-

1. Binds human recombinant TNF alpha, but not human lymphotoxin (TNF beta) or human interferon. Similarly MAb 1 does not cross-react with recombinant murine TNF (Fig.1).
2. MAb 1 is of the immunoglobulin type IgG1, K with an apparent affinity of 4.4 x 10^{-9} moles/litre (Fig. 2).
3. MAb neutralises the cytotoxic effect of recombinant human TNF on WEHI-164 mouse fibrosarcoma cells in culture. One microgram of MAb 1 neutralizes approximately 156.25 units of TNF in vitro (Fig. 3).
4. MAb 1 neutralises the tumour regression activity of TNF in the following mouse tumour models in vivo; WEHI-164 subcutaneous solid tumour, the Meth A subcutaneous solid tumour and the Meth A ascites tumour (Figs. 4, 5 and 9).
5. MAb1 prevents cerebral damage caused by human TNF in mice infected with malarial parasites.
6. In radioreceptor assays MAb 1 prevents binding of TNF to receptors on WEHI-164 cells (Table 3).

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7. MAb 1 inhibits the induction of procoagulant activity (tissue factor) on cultured bovine aortic endoth lial cells (Fig 6).

8. MAb 1 reduces the uptake of 1251 fibrinogen into tumours of mice treated with TNF (Fig. 7).

9. MAb 1 competes for binding of 1251 TNF and thus shares an overlapping epitope with the following monoclonal antibodies: 21, 25, 32, 47, 54 and 37.

10 MAb 1 does not compete for binding of 1251 TNF with the following monoclonal antibodies: 11, 12, 42, 53, 31 and 20 (Fig. 8).

TABLE 3

RADIORECEPTOR ASSAY: INHIBITION OF TNF BINDING TO WEHI-164 CELLS BY MAb 1		
TREATMENT		% SPECIFIC BINDING
MAb 1	1/10	0
	1/100	21
	1/1,000	49
	1/10,000	73
	1/100,000	105
cold TNF (ng/tube)		
	10,000	0
	5,000	0
	1,000	0
	500	10
	100	11
	10	64
	1	108
	0	100

[0036] Mab 32 is an IgG2b,K antibody with an affinity for human TNF alpha of 8.77 $\times 10^{-9}$ moles/litre as determined by Scatchard analysis. This monoclonal antibody does not react with either human TNF beta (lymphotoxin) or mouse TNF alpha.

[0037] As shown in Figure 3 MAb 32 does not inhibit TNF cytoxicity in vitro as determined in the WEHI-164 assay.

[0038] Monoclonal antibody 32 variably enhances TNF-induced tumour regression activity against WEHI-164 fibrosarma tumours implanted subcutaneously into BALB/c mice at a TNF dose of 10μg/day (see Figs. 10 and 11). This feature is not common to all monoclonal antibodies directed against TNF (Fig. 9) but resides within the binding site specificity of MAb 32 (Fig. 8) which may allow greater receptor mediated uptake of TNF into tumour cells (see Table 4).

TABLE 4

BINDING OF TNF TO RECEPTORS ON WEHI-164 CELLS IN THE PRESENCE OF MAb 32		
	% BINDING ^{125} I-TNF	
MAB DILUTION	CONTROL MAB	MAB 32
1/10	36	141
1/100	74	88
1/1000	101	83
1/10,000	92	82
1/100,000	97	93

[0039] Enhancement of TNF activity by MAb 32 at lower doses of TNF is such that at least tenfold less TNF is required to achieve the same degree of tumour regression (see Fig. 11 and 18). The results for day 1, 2.5ug and 1ug TNF and day 2, 5ug, 2.5ug and 1ug are statistically significant in a t-test at p <.01 level. This level of enhancement also increases the survival rate of recipients since the lower dose of TNF used is not toxic. Fig. 19 shows that univalent Fab fragments of MAb 32 also cause enhancement of TNF-induced tumour regression in the same manner as whole MAb 32 (see below).

[0040] MAb 32 inhibits the expression of clotting factors on endothelial cells normally induced by incubation of the

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cultured cells with TNF (see Fig. 6). This response may be mediated by a previously unidentified TNF receptor which is distinct to the receptor found on other cells.

[0041] Conversely, MAb 32 enhances th *in vivo* activation of coagulation within the tumour bed as shown by the incorporation of radiolabelled fibrinogen (Fig. 7). This may be due to activation of monocytes/macrophage procoagulant and may provide further insight into the mechanism of TNF-induced tumour regression.

[0042] The results obtained with MAb 32 are shown in comparison to other anti-TNF MAbs in Table 2.

[0043] The ability of MAb 32 and MAb 47 to inhibit the binding of TNF to endothelial cells was also assessed. Bovine aortic endothelial (BAE) cells (passage 11) were plated in 24-well culture dishes (Corning) which had been pre-coated with gelatin (0.2%) and grown to confluence in McCoys 5A (modified) medium supplemented with 20% foetal calf serum. For the radio-receptor assay all dilutions (of cold TNF and MAbs) were made in this medium. The BAE cells were incubated for one hour in the presence of either cold TNF (0 to 100ng) or MAb (ascites globulins diluted 1/100 to 1/100,000) and iodinated TNF (50,000 cpm). At the end of this time the medium was withdrawn and the cells washed before being lysed with 1 M sodium hydroxide. The cell lysate was then counted for bound radioactive TNF. Specific binding of labelled TNF to the cells was then determined.

[0044] The results obtained in this assay with MAb 32, MAb 47 and a control MAb are set out in Figure 12.

[0045] The results obtained in the clotting assay using BAE cells cultured in the presence of TNF and anti-TNF MAb correlate with the results obtained in the BAE radioreceptor assay i.e. MAbs which inhibit the induction of clotting factors on the surface of endothelial cells (as shown by the increase in clotting time compared to TNF alone) also inhibit the binding of TNF to its receptor. This is exemplified by MAbs 32 and 47.

[0046] MAb 32, which does not inhibit TNF binding to WEHI-164 cells, does inhibit binding of TNF to endothelial cells. This result provides support for the hypothesis that distinct functional sites exist on the TNF molecule and that these sites interact with distinct receptor subpopulations on different cell types. Thus ligands which bind to defined regions of TNF are able to modify the biological effects of TNF by limiting its binding to particular receptor subtypes.

[0047] As shown in Figure 12 MAb 47 is a particularly potent inhibitor of TNF interaction with endothelial cells, the percentage specific binding at a dilution of 1/100 to 1/10,000 being effectively zero

RECEPTOR BINDING STUDIES OF HUMAN TNF COMPLEXED WITH MAB 32 ON HUMAN CARCINOMA CELL LINES IN VITRO

[0048] MAb 32 has been shown to enhance the anti-tumour activity of human TNF. The mechanisms behind the enhancement may include restriction of TNF binding to particular (tumour) receptor subtypes but not others (endothelial) with subsequent decrease in TNF toxicity to non-tumour cells. This mechanism does not require enhanced uptake of TNF by tumour cells in *in vitro* assays. In addition, MAb 32 also potentiates the binding of human TNF directly to TNF receptors on certain human carcinoma cell lines.

MATERIALS AND METHODS

[0049] The following human carcinoma cell lines have been assayed for enhanced receptor-mediated uptake of TNF in the presence of MAb 32: B10, CaCo, HT 29, SKC01 (all colon carcinomas), 5637 (Bladder carcinoma), MM418E (melanoma), IGR3 (melanoma), MCF 7 (breast carcinoma). The cells were propogated in either RPMI-1640 (MM418E) DMEM (CaCo and IGR 3) or Iscoves modified DMEM (B10, HT 29, SK01, S637, MCF 7) supplemented with 10% foetal calf serum, penecillin/streptomycin and L-glutamine. Receptor assays were performed as previously described for endothelial cells except that the incubation time with iodinated TNF was extended to 3 hours for all but the B10 cells for which the radiolabel was incubated for 1 hour.

RESULTS

[0050] Enhanced TNF uptake was observed in the presence of MAb32 by the melanoma cell lines tested MM418E and IGR 3 (Figs. 13 and 14), the bladder carcinoma 5637 (Fig. 15), and the breast carcinoma MCF 7 (Fig. 16). MAb 32 did not affect TNF-receptor interaction in any of the other cell lines as shown by B 10 (Fig. 17) MAb 47, which has been shown to inhibit TNF binding to WEHI-164 cells and endothelial cells, and which also inhibits TNF-mediated tumour regression was found to markedly inhibit TNF binding to all the cell lines tested (Figs. 13-17).

CONCLUSIONS

[0051] Receptor binding analyses have indicated a second mechanism whereby MAb 32 may potentiate the anti-tumour activity of TNF This second pathway for enhancement of TNF results from increased uptake of TNF by tumour all receptors in the presence of MAb 32.
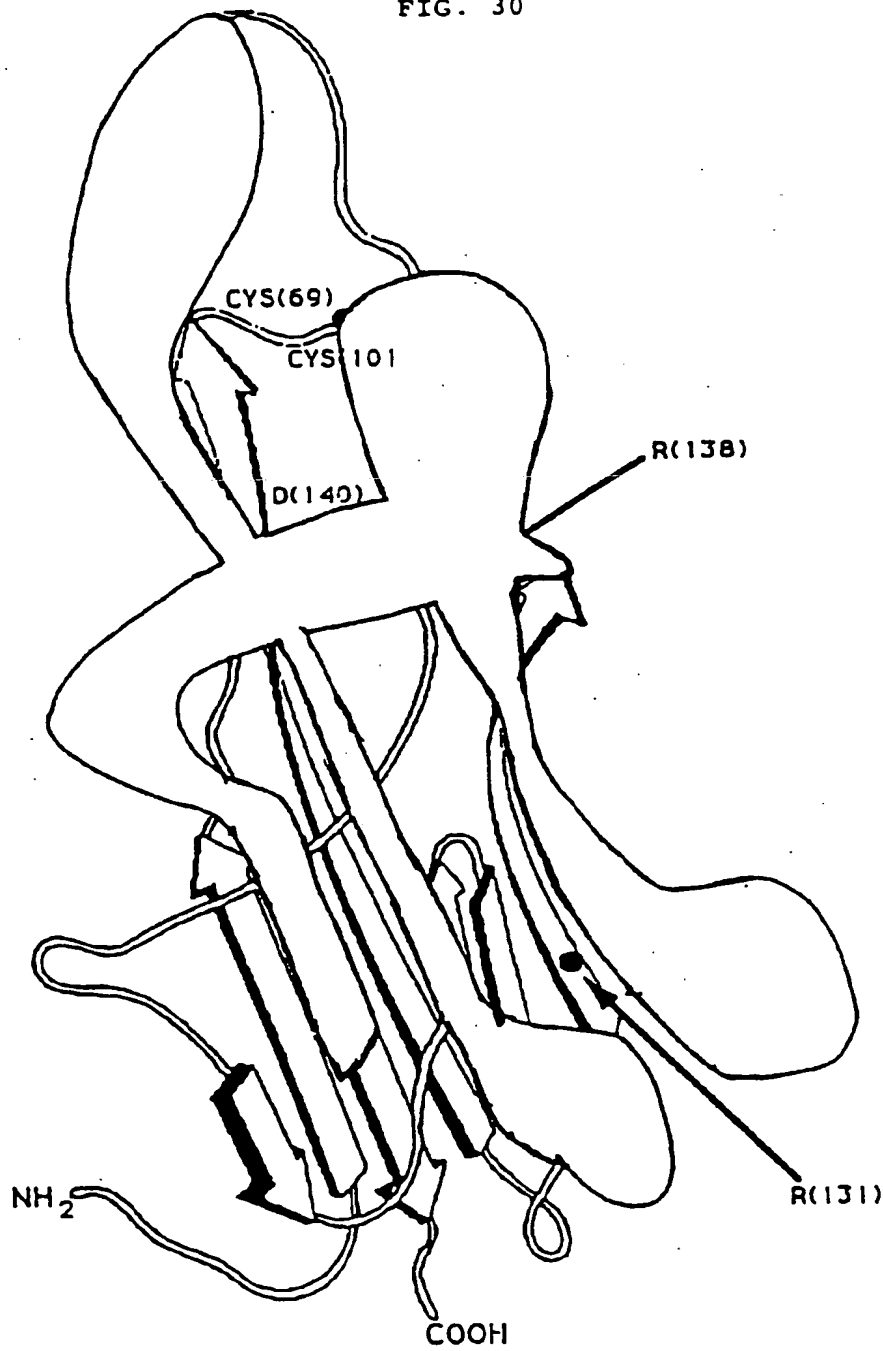
FIG. 30

NH₂

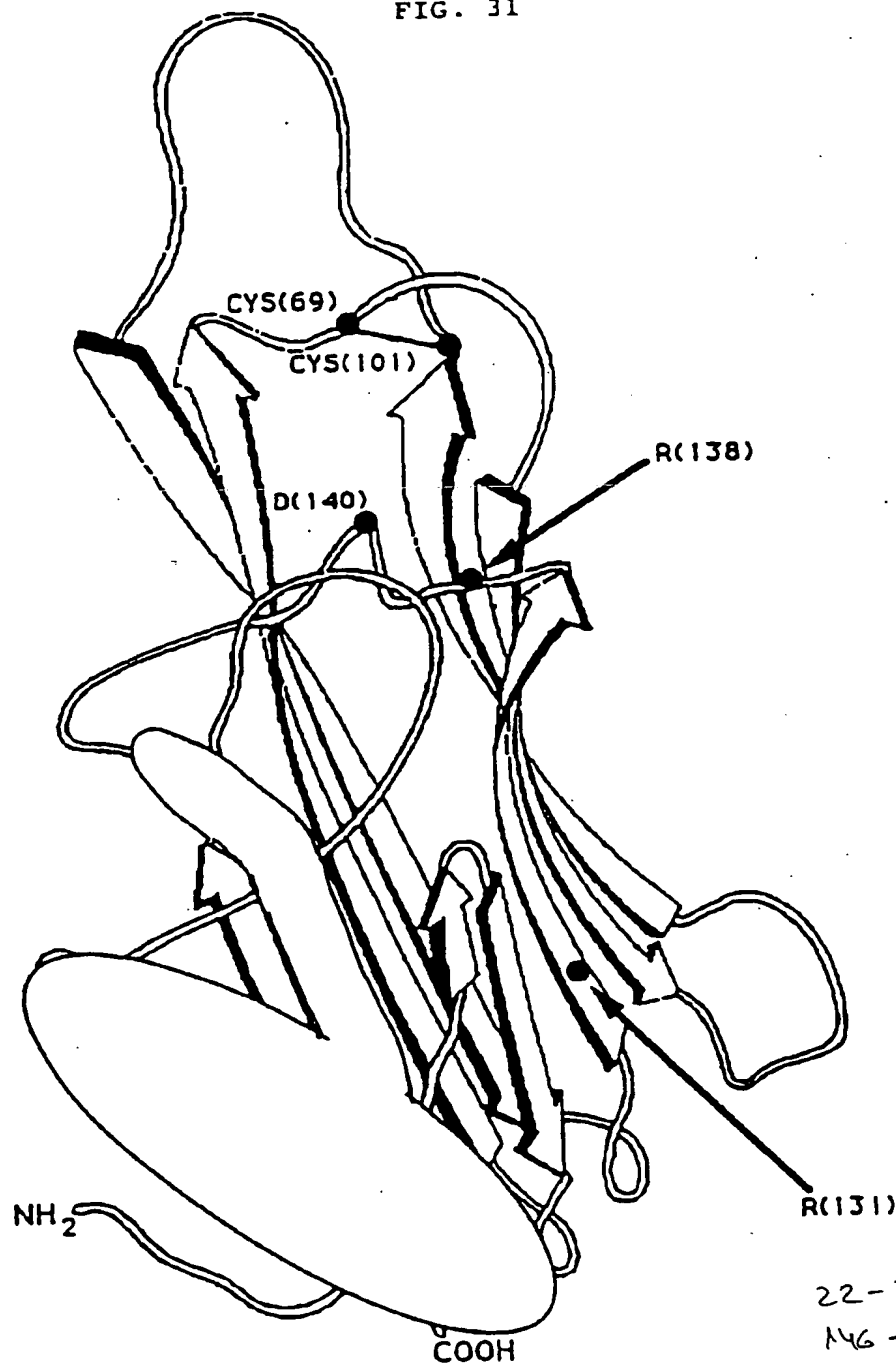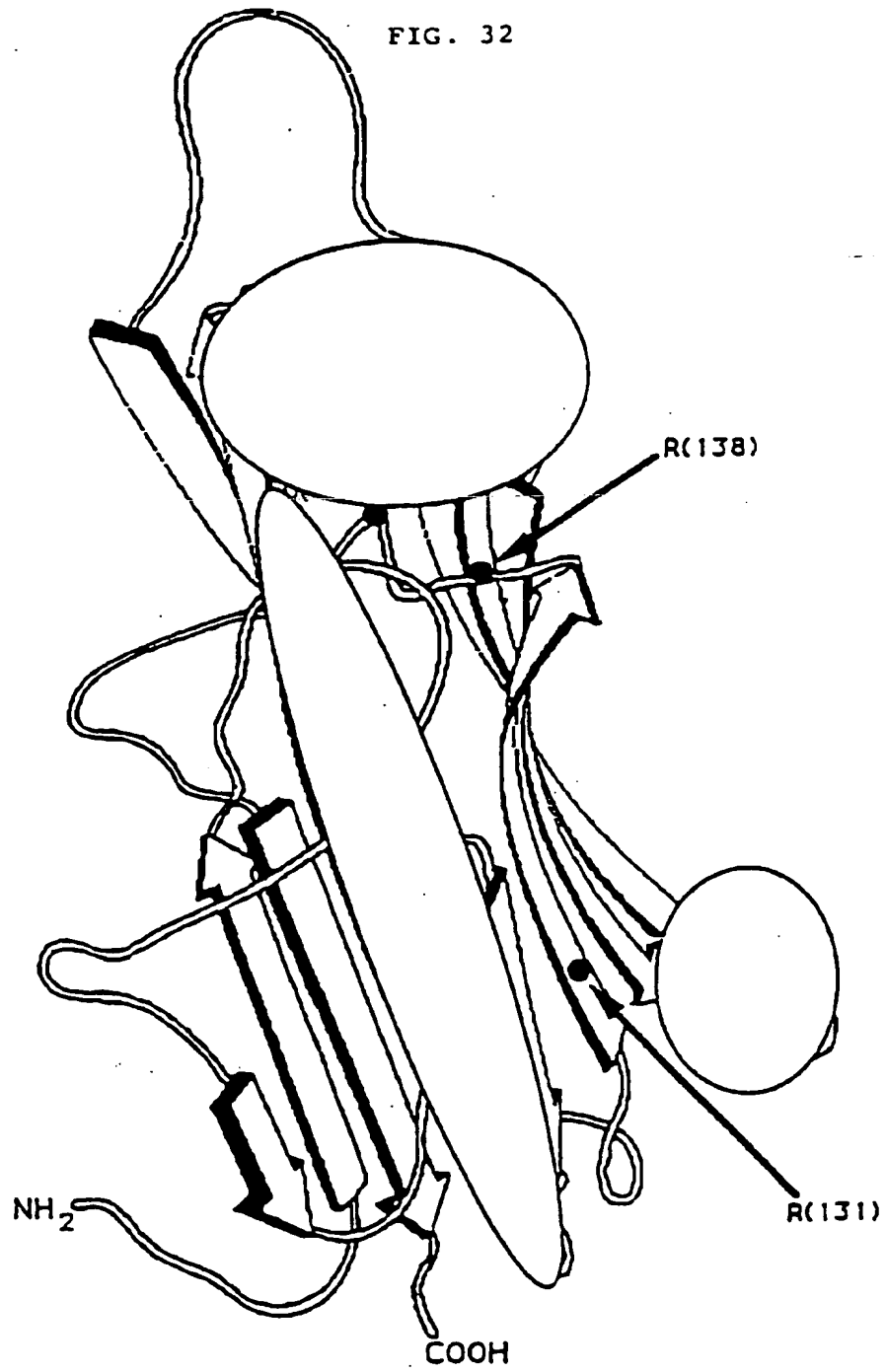COOH

CYS(69)
CYS 101
D(140)
R(138)
R(131)

22-40
68-97
105-128
135-155

FIG. 31

CYS(69)

CYS(101)

R(138)

D(140)

R(131)

NH₂

COOH

22-31
146 - 157

FIG. 32

R(138)

R(131)

NH$_2$

COOH

FIG. 33